



## The Silence of Genes in Transgenic Plants

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In genetically modified plants, the introduced transgenes are sometimes not expressed. They can be silenced. Transgenes can also cause the silencing of endogenous plant genes if they are sufficiently homologous, a phenomenon known as co-suppression. Silencing occurs transcriptionally and post-transcriptionally but silencing of endogenous genes seems predominantly post-transcriptional. If viral transgenes are introduced and silenced, the post-transcriptional process also prevents homologous RNA viruses from accumulating; this is a means of generating virus-resistant plants. A major goal of current research is to dissect the mechanism(s) of these sequence-homology-dependent gene silencing phenomena. Various factors seem to play a role, including DNA methylation, transgene copy number and the repetitiveness of the transgene insert, transgene expression level, possible production of aberrant RNAs, and ectopic DNA–DNA interactions. The causal relationship between these factors and the link between transcriptional and post-transcriptional silencing is not always clear. In this review we discuss various observations associated with gene silencing and attempt to relate them.

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### INTRODUCTION

Analysis of the large collection of genetically modified plants generated in recent years has expanded our knowledge of physiological processes and gene regulation mechanisms tremendously. However, transgenes do not always behave as expected. This has revealed the existence of hitherto unknown cellular processes. There is considerable variation in the expression of transgenes in individual transformants which is not due to differences in copy number. Thus, gene activity is not exclusively determined by the strength of the promoter which controls transcription; epigenetic effects also influence expression levels. This sometimes leads to gene inactivation either by blocking transcription or by inhibiting mRNA accumulation. Until now, gene silencing has been a confusing field of research. Most of us stumbled upon this phenomenon fortuitously by analysing transgenic plants. The mechanisms by which silencing is achieved are still poorly understood. Despite the different silencing systems being examined, several cases of gene silencing have features in common which gives us insight into the factors involved.

Gene silencing also occurs in untransformed plants where it reduces expression of endogenous genes. A number of mutations in various plant species appear to result from epigenetic gene silencing. For example, paramutation in maize (Brink, 1973) and tomato (Hageman, 1993) probably involve gene–gene interactions. Although paramutation was known long before the discovery of transgene-mediated silencing, it is only recently that the underlying molecular mechanisms have become apparent. Several features re-

semble those associated with transgene-mediated silencing. Because of space limitations, we will only discuss the silencing of transgenes and endogenous genes in transgenic plants. A detailed description of paramutation and related phenomena in untransformed plants can be found in other reviews (Matzke and Matzke, 1993; Patterson and Chandler, 1995) and some recent articles (Das and Messing, 1994; Hollick *et al.*, 1995; Patterson *et al.*, 1995; Ronchi, Petroni and Tonelli, 1995). Various aspects of transgene-mediated silencing discussed here can also be found in other reviews (Finnegan and McElroy, 1994; Flavell, 1994; Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe and English, 1996; Meyer, 1996) and books (Paszowski, 1994; Meyer, 1995; Grierson, Lycett and Tucker, 1996).

### SILENCING OF TRANSGENES

Various possibilities have been raised to explain variation in transgene expression levels among transformants that is independent of copy number. All these imply that integrated transgenes cannot be regarded as independent transcription units. Transgenes, often as part of the *Agrobacterium tumefaciens* T-DNA, integrate at different chromosomal locations. If they become inserted into euchromatin, in a transcriptionally active region (Koncz *et al.*, 1989; Herman *et al.*, 1990; Kertbundit *et al.*, 1991), expression may be influenced by regulatory sequences of nearby host genes. If they insert in or near repetitive DNA or heterochromatin, they can be inactivated (Pröls and Meyer, 1992). Another important factor associated with gene silencing is the number of transgenes per integration site. The T-DNA transfer system can insert two or more T-DNAs at the same chromosomal site. These T-DNAs can be arranged 'head-

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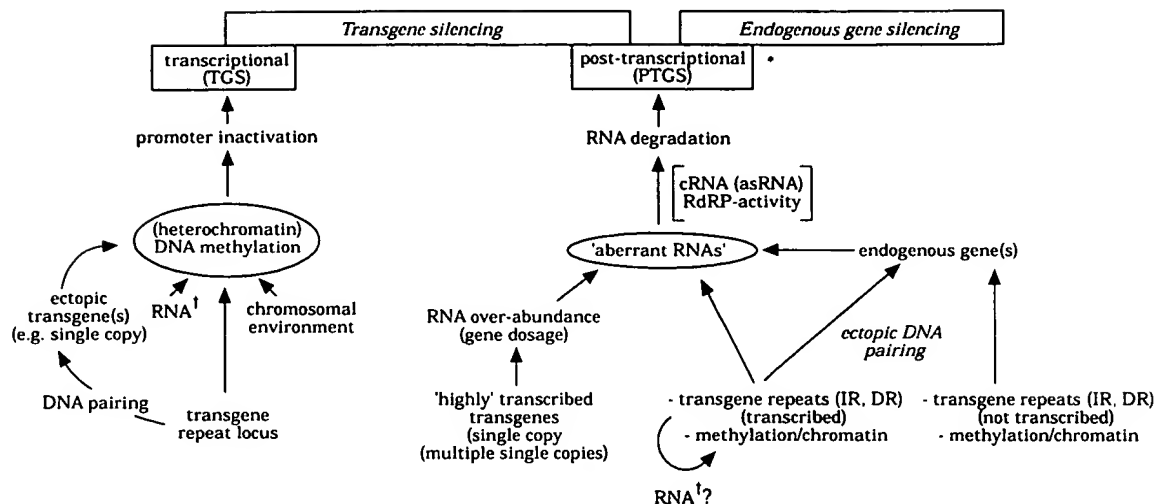


FIG. 1. Gene silencing pathways in transgenic plants. Various, seemingly conflicting, observations are combined to illustrate their possible inter-relations (see text for details). A key feature of transcriptional silencing of transgenes is DNA methylation (encircled) and possibly, the formation of heterochromatin, by which promoters become inactive. Models of post-transcriptional silencing of transgenes and of endogenous genes are speculative. The key features considered are: the production of aberrant transcripts (encircled), the activity of a host encoded RNA-directed RNA polymerase (RdRP) and production of complementary RNA (cRNA or antisense RNA). The ways aberrant RNAs might be produced are indicated in the bottom right half of the figure. Note that transcriptionally inactive and promoterless transgenes are assumed to trigger PTGS of the endogenous gene via an ectopic interaction (bottom far right). \* All endogenous genes tested thus far are post-transcriptionally silenced by transgenes which contain the corresponding coding region, except in one case where an homologous *cab140* promoter was used to drive a *tms2* transgene which led to a reduced transcription of the endogenous *cab* gene (Brusslan *et al.*, 1993). † In one case it has been reported that viroid RNA in plants triggered the methylation of the corresponding transgene sequence in the genome (Wassenegger *et al.*, 1994).

to-tail' as a direct repeat (DR), and 'head-to-head' or 'tail-to-tail' as an inverted repeat (IR). Transgenes of T-DNAs that are organized as IRs often show low expression (Jones *et al.*, 1987; Jorgensen, Snyder and Jones, 1987) indicating that the genes are silenced to some degree.

There are two kinds of gene silencing. Firstly, transcriptional gene silencing (TGS), which results from promoter inactivation; and secondly, post-transcriptional gene silencing (PTGS) which occurs when the promoter is active but the mRNAs fail to accumulate. Even though this clear difference suggests two distinct silencing mechanisms, the two seem related, in particular when one invokes interactions between homologous DNA sequences. This notion is inspired by observations that DNA methylation, which is often associated with TGS, is sometimes also found associated with PTGS (Hobbs, Warkentin and Delong, 1993; Ingelbrecht *et al.*, 1994; Smith *et al.*, 1994; English, Mueller and Baulcombe, 1996). The scheme presented in Fig. 1 attempts to connect the various features of TGS and PTGS. Although some of the proposed interactions and models are highly speculative, this scheme functions as a framework for the discussions below.

#### Silencing-associated DNA methylation

As methylation in eukaryotes causes gene inactivation (Bird, 1992; Martienssen and Richards, 1995) it is not surprising that it is also found associated with transgene silencing (Matzke *et al.*, 1989; Linn *et al.*, 1990; Kilby, Leyser and Furner, 1992; Assaad, Tucker and Signer, 1993; Hobbs *et al.*, 1993; Vaucheret, 1993; Ingelbrecht *et al.*, 1994; Meyer, Niedenhof and Ten Lohuis, 1994; Smith *et al.*,

1994; Vaucheret, 1994). In plant DNA, 5-methyl-cytidine is found in CpG and CpNpG symmetrical sites but also in non-symmetrical sites (Ingelbrecht *et al.*, 1994; Meyer *et al.*, 1994; Park *et al.*, 1996), thus essentially any cytidine in the DNA can be methylated.

TGS is often associated with heavily methylated and inactive promoter sequences (Meyer, Heidmann and Niedenhof, 1993; Neuhuber *et al.*, 1994; Park *et al.*, 1996). The effect of methylation on gene expression in other parts than the promoter is less obvious. Although methylation of the coding region generally has no detectable effects on transcription (Hobbs *et al.*, 1993; Ingelbrecht *et al.*, 1994; Smith *et al.*, 1994; English *et al.*, 1996) in some cases it appears to be involved in PTGS (see below).

Gene silencing is usually detected when the system has reached a steady-state condition. This means that nothing is known about the events shortly after the transgenes have been inserted into the genome. Therefore, it remains unresolved whether methylation establishes silencing, or whether it is a response to a change in chromatin structure and that it now functions epigenetically to maintain and transmit the silent state. Some of the factors that might be involved in *de novo* methylation of transgene sequences are considered below.

**Chromosomal environment of the transgene.** When a transgene integrates into a chromosomal region that is heavily methylated and/or repetitive, it is silenced (Pröls and Meyer, 1992). This suggests that the character of the flanking DNA, methylated or heterochromatin, is imposed upon the inserted transgenes. However, a transgene integrated into hypomethylated DNA can also be transcriptionally inactivated (Pröls and Meyer, 1992; Meyer *et al.*,

*al.*, 1993). This has been observed with a maize *Al* gene in petunia whereby only the inserted DNA was methylated (Meyer and Heidmann, 1994). This *de novo* methylation of foreign DNA is thought to be a cellular defence response against the potential harmful activity of this DNA (Doerfler, 1995). How DNA is recognized as being 'foreign' is unknown, but it might be related to a different adenine-thymine (AT) content relative to that of the flanking DNA (Salinas *et al.*, 1988; Matassi *et al.*, 1989; Meyer and Heidmann, 1994; Elomaa *et al.*, 1995).

**Repeat-induced DNA methylation.** Transgenes of T-DNAs which are inserted as a DR or an IR have a tendency to become inactivated. This is frequently associated with DNA methylation (Hobbs, Kpodar and DeLong, 1990; Kilby *et al.*, 1992; Assaad *et al.*, 1993; Hobbs *et al.*, 1993; Vaucheret, 1993; Matzke *et al.*, 1994; Stam and Kooter, unpubl. res.). The trigger of this *de novo* methylation is unknown but is probably a response to the repetitive nature of the locus. But how? In the case of IRs it might be the ability to create a cruciform which is a good substrate for DNA methyltransferases (Laayoun and Smith, 1995). In general, the more copies a locus contains, the stronger the inactivation (Matzke *et al.*, 1994). Assaad *et al.* (1993) were able to follow recombination derivatives of a transgene locus that contained repetitive sequences. Predominantly, the multi-gene recombinants showed silencing. These cases of repeat-induced gene silencing (RIGS) resemble phenomena in other eukaryotes, such as repeat-induced point mutation (RIP) in *Neurospora crassa* (Singer, Marcotte and Selker, 1995; Singer and Selker, 1995) and methylation-induced pre-meiotically (MIP) in *Ascobolus immersus* (Rhounim, Rossignol and Faugeron, 1992; Rossignol and Faugeron, 1995). In these fungi, inactivation of tandemly duplicated genes is very efficient and there is evidence to suggest that it is mediated by DNA-DNA pairing (Selker and Garrett, 1988).

**DNA-DNA interactions and gene silencing in trans.** A transgene locus that is silenced can silence homologous transgenes at ectopic loci (Matzke *et al.*, 1989; Vaucheret, 1993; Matzke *et al.*, 1994). When the silencing locus is methylated, the target locus also becomes methylated. In the case of homologous promoters this transfer of methylation can lead to transcriptional inactivation. This has been described in detail for a potent silencer locus, the 271 transgene locus, which contains antisense nitrite reductase genes driven by a strong CaMV-35S promoter (Vaucheret, 1993; Elmayan and Vaucheret, 1996; Park *et al.*, 1996). This silencing locus contains multiple copies and appears very efficient in silencing homologous sequences elsewhere in the genome which are then methylated. When and how this interaction occurs, and how it leads to methylation is unknown, but some of the possibilities are discussed by Matzke and Matzke (1995).

Methylation of the target genes is erased after crossing out the silencing locus. This does not happen immediately but occurs gradually in successive generations (Matzke and Matzke, 1991) or even during a plant's lifetime (Vaucheret, 1994). Thus, to maintain the fully inactive state, the silencing locus needs to be present permanently, suggesting regular cross-talk between the homologous sequences. Cross-talk

between homologous transgene sequences might be enhanced in homozygous plants. An inactive single copy transgene is even able to inactivate an active allele *in trans*, probably by an allelic interaction (Meyer *et al.*, 1993). This and other results (see below) indicate that even with single copy insert transformants, which usually give the least problems concerning stable transgene expression, one occasionally encounters gene inactivation in plants homozygous for the transgene. Potentially, this may cause problems in breeding programmes where inbred lines are used. It occurs quite often that transgenes in hemi- and homozygous plants behave differently. In many cases silencing is only observed or is severely enhanced in homozygous plants (e.g. De Carvalho *et al.*, 1992; Hart *et al.*, 1992; Dehio and Schell, 1994; Brandle *et al.*, 1995; Vaucheret *et al.*, 1995; Elmayan and Vaucheret, 1996; Howie *et al.*, 1996). It is interesting to note that silencing also occurs in haploid plants that were generated from anthers from non-silenced hemizygous plants (De Carvalho *et al.*, 1992; Elmayan and Vaucheret, 1996). These results indicate that an allelic interaction between transgene loci is not necessary in homozygous plants for the induction of silencing, merely the gene dosage per genome is important.

#### POST-TRANSCRIPTIONAL SILENCING OF TRANSGENES AND HOST GENES

Transcriptional silencing as a result of promoter inactivation by DNA methylation and/or heterochromatinization is conceptually straightforward, even though many of the underlying molecular events need to be worked out. It seems more complicated when gene silencing results from a post-transcriptional process (Fig. 1, right-hand side). In this case, promoters are active and the genes transcribed, but mRNA fails to accumulate. The transgene-induced PTGS mechanism affects expression of the transgenes and of endogenous genes with which they share a considerable degree of sequence identity. The latter case is also known as co-suppression because the endogenous genes and the transgenes are silenced. Co-suppression was first observed with genes involved in flower pigmentation (Napoli, Lemieux and Jorgensen, 1990; Van der Krol *et al.*, 1990) and in tomato fruit ripening (Smith *et al.*, 1990). Silencing of the chalcone synthase (*chs*) genes in petunia flowers occurs post-transcriptionally (Van Blokland *et al.*, 1994; Metzlaiff, O'Dell, and Flavell, 1996). Using homologous sense transgenes, the expression of many endogenous genes has been suppressed. This approach is now frequently used to study gene function as inactivation of endogenous genes can result in mutant phenotypes (Fig. 2).

#### Characteristics of PTGS

The PTGS mechanism appears to act on any RNA that is homologous to the transgene that has activated it, thus from transgenes, endogenous genes and from transiently expressed genes (Hobbs *et al.*, 1993; English *et al.*, 1996). Also, viral RNAs are attacked when transgenes are used that contain viral sequences (De Haan *et al.*, 1992; Lindbo *et al.*, 1993; Mueller *et al.*, 1995). The same holds for

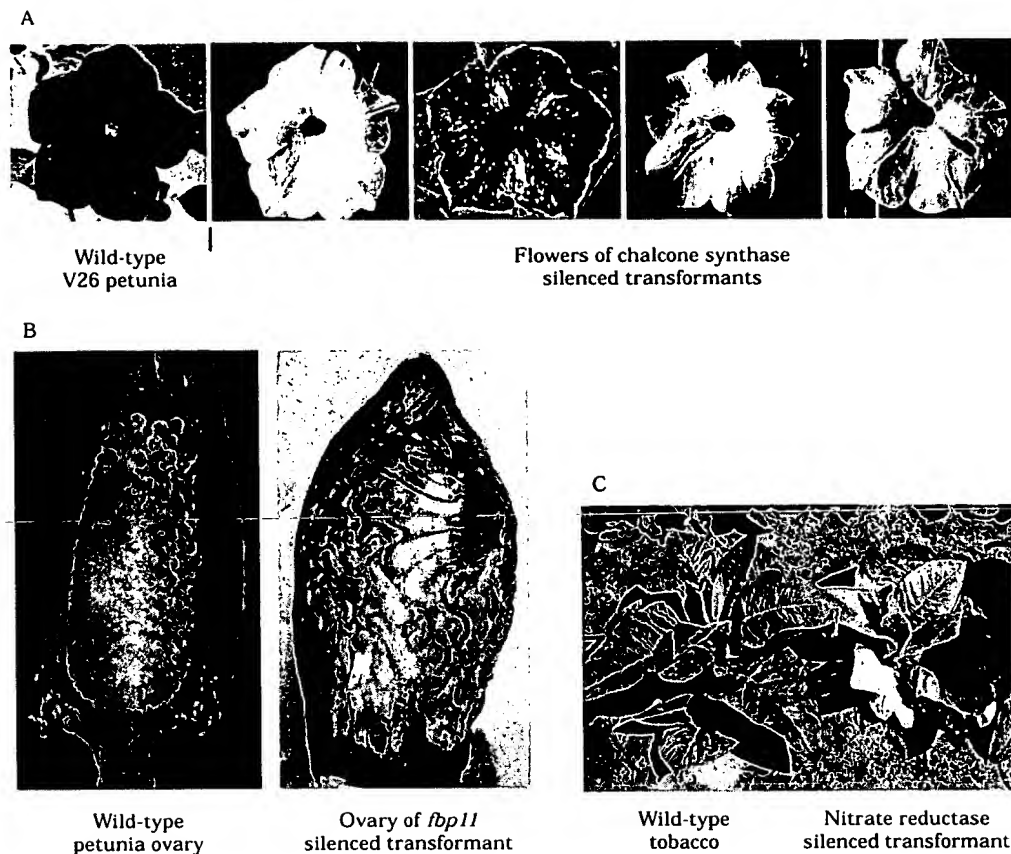


FIG. 2. Phenotypic alterations caused by transgene-induced silencing of endogenous genes. A, Silencing of the anthocyanin biosynthesis gene chalcone synthase (*chs*) in petunia corollas by *chs* sense transgenes. The degree and extent of silencing varies among independent transformants resulting in various pigmentation patterns (Van Blokland *et al.*, 1994; Stam and Kooter, unpubl. res.). On the left is a wild-type V26 flower. The cause of the patterned silencing is unknown. B, Silencing of *fbp11*, a MADS box gene, in petunia transformants containing CaMV-35S driven sense *fbp11* transgenes. This results in aberrant ovule development. On the left is a wild-type ovary; on the right that of the *spaghetti* mutant (Photograph kindly provided by Gerco Angenent; For details, see Angenent *et al.*, 1995). C, Silencing of nitrate reductase expression in tobacco by CaMV-35S promoter driven sense nitrate reductase transgenes which results in chlorotic plants (right). (Photograph kindly provided by Hervé Vaucheret. Reprinted by permission from Molecular & General Genetics. Copyright 1994, Springer Verlag; For details, see Dorlhac de Borne *et al.*, 1994).

engineered chimaeric viruses which contain non-viral sequences. Transformants carrying silenced *uidA*, *np11* transgenes, or polygalacturonase trans- and endogenous genes, do not accumulate chimaeric viruses that carry sequences of these genes (English *et al.*, 1996). The 'foreign' sequence in the viral RNA acts as a target for the degradation machinery. Interestingly, in the case of *uidA*, only viruses which carried the 3' region of the *uidA* coding region did not accumulate. Thus not every sequence can act as a target for RNA degradation (see below).

The proportion of transformants in which transgenes and/or homologous host genes are silenced varies, but can be very high (Elmayan and Vaucheret, 1996; Jorgensen *et al.*, 1996). The reasons for this are not known exactly, but they seem to be related to transgene expression levels and the organization of the T-DNA inserts. Moreover, silencing does not always occur in every cell of a particular tissue. In the case of anthocyanin gene silencing, this is clearly visible by the various flower coloration patterns (Van der Krol *et al.*, 1990; Van Blokland *et al.*, 1994; Jorgensen, 1995;

Jorgensen *et al.*, 1996) (Fig. 2A). In some systems the PTGS is developmentally regulated. Silencing of *uidA* (Elmayan and Vaucheret, 1996), chitinase (Kunz *et al.*, 1996) and of  $\beta$ -1,3-glucanase transgenes in tobacco (De Carvalho Niebel *et al.*, 1995), does not occur in young seedlings but starts between 2 and 6 weeks after germination, depending on the expression level of the transgenes. A similar meiotically reversible silencing was observed with *rolB* transgenes in *Arabidopsis* (Dehio and Schell, 1994). Taken together, the PTGS mechanism(s) must not only account for the enhanced turnover of specific transcripts but also for the developmental and spatial control of this process.

#### Possible PTGS mechanisms

The hallmark of PTGS is the reduced accumulation of specific transcripts. Whether the reduction results from RNA instability, from activation of a sequence-specific RNA degradation process, or a combination of both, is unknown. The right-hand side of Fig. 1 gives an overview of

several possibilities in an attempt to relate different PTGS features. These include a role for transgene expression level, for the structure and methylation of a multicopy locus, and whether or not the nucleus contains an endogenous homologue. The latter is important because the mRNA elimination process may be activated by means of an interaction of the transgene locus with the endogenous gene(s) (Van Blokland *et al.*, 1994) which might only be possible with particular transgene loci.

**Role of transgene expression level.** An attractive model explaining PTGS invokes an effect of highly expressed transgenes. In this model, the transgenes produce so much RNA that the level exceeds a critical threshold thereby triggering a mechanism that specifically removes all homologous RNAs irrespective of their source. This RNA threshold model gained support from observations whereby PTGS was correlated with highly expressed transgenes conferring viral resistance (Lindbo *et al.*, 1993; Smith *et al.*, 1994; Goodwin *et al.*, 1996). Plants containing highly transcribed transgenes did not accumulate the transgenic mRNAs and were also resistant to infection by an homologous RNA virus (Smith *et al.*, 1994; Goodwin *et al.*, 1996). Plants in which the transgenes were transcribed at lower levels accumulated transgenic RNA normally, but were also sensitive to a viral infection. Sometimes a virus infection is needed to induce the PTGS mechanism and resistance. This result is in line with reaching the hypothetical RNA threshold level (Lindbo *et al.*, 1993). The threshold model may also explain PTGS of highly expressed *uidA* transgenes in tobacco (Elmayan and Vaucheret, 1996) and of single copy *chs* transgenes in petunia (Jorgensen *et al.*, 1996). Also studies by Metzlaiff *et al.* (1996) of *chs* co-suppression in petunia transformants suggest that silencing requires active transgenes.

Not all cases of PTGS are associated with highly expressed transgenes. For example, the degree of viral resistance is not always directly related to viral transgene expression (Mueller *et al.*, 1995; English *et al.*, 1996; see below and reviews on strategies and mechanisms of viral resistance by Pang, Slightom and Gonsalves, 1993; Baulcombe, 1994, and Dougherty and Parks, 1995). Also silencing of the *chs* genes in petunia is not always associated with highly transcribed *chs* transgenes (Van Blokland *et al.*, 1994). This suggests that the absolute level of normal mRNAs is not important. It might be more crucial to have sufficient amounts of an RNA that is qualitatively distinct from the regular mRNA. These so-called aberrant RNAs are thought to be required for activating the PTGS mechanism (Dougherty and Parks, 1995; Metzlaiff *et al.*, 1996).

**PTGS and methylated transgenes.** PTGS is frequently associated with methylated transgenes (Hobbs *et al.*, 1990, 1993; Ingelbrecht *et al.*, 1994; Smith *et al.*, 1994; English *et al.*, 1996). The PTGSed *nptII* genes described by Ingelbrecht *et al.* (1994) were methylated up- and downstream of the coding region. Also Smith *et al.* (1994) noticed that the viral transgenes of the virus-resistant plants were methylated more than those of the sensitive plants. Despite this methylation, the promoters were active and the genes transcribed, but whether the transcripts were normal is unknown. In *Ascorbolus immersus*, transcription of meth-

ylated genes gives rise to truncated transcripts (Barry, Faugeron and Rossignol, 1993). Although this was suggested to result from premature termination of transcription, a specific degradation of the RNA part that is transcribed from the methylated DNA region cannot be ruled out. Indeed Ingelbrecht *et al.* (1994) showed that post-transcriptionally silenced *nptII* genes in tobacco were transcribed beyond methylated restriction sites at the 3' end of the gene.

Studies by English *et al.* (1996) provided more direct evidence for a role of DNA methylation in PTGS. The PTGSed *uidA* genes in the tobacco plants described by Hobbs *et al.* (1990, 1993) were more methylated near the 3' end of the gene which included the poly-adenylation region. Importantly, this 3' region was also needed to prevent infection by the PVX-*uidA* chimeric viruses, as if this part of the RNA is the target for the degradation of RNA. Thus, there seems to be a link between DNA-methylation and the RNA-based silencing mechanism (English *et al.*, 1996).

Although in some cases transgene PTGS is associated with methylation, there is no evidence that the post-transcriptionally silenced endogenous genes are methylated (Hart *et al.*, 1992; Stam and Kooter, unpubl. res.), despite the fact that the transgenes contain coding regions which are potentially able to induce methylation of a target locus. Where it has been examined, the promoters of post-transcriptionally silenced endogenous genes are normally active and the genes are transcribed at wild-type levels (Van Blokland *et al.*, 1994; De Carvalho Niebel *et al.*, 1995; Kunz *et al.*, 1996). However, there is one example where transcription of an endogenous gene, *cab140* in *Arabidopsis*, is decreased (five-fold) due to the presence of a *cab140* promoter-driven transgene (Brusslan *et al.*, 1993; Brusslan and Tobin, 1995). This reduced transcription is probably the result of an epigenetic change of the promoter caused by the homology between the promoters of the transgene and the endogenous genes. The molecular basis of the silencing is not yet known but methylation does not seem to be involved (Brusslan *et al.*, 1993).

**RNA-directed DNA methylation (RdDM).** There is one study that provides evidence for a role of RNA in *de novo* methylation of the homologous DNA (Wassenegger *et al.*, 1994). In tobacco transformants it was found that transgenes composed of three or four tandemly arranged viroid cDNAs in sense or antisense orientation and driven by the CaMV-35S promoter were methylated. It was argued that this methylation was caused by the viroid RNAs. However, it cannot be ruled out that methylation of the three and four-copy transgene constructs was due to the repetitive nature of the transgenes (see above). The demonstration that a two-copy and a monomeric viroid cDNA construct, which were unmethylated, became methylated after the plant was infected with viroid RNA and replication had taken place, indicate that the viroid RNA is responsible for the methylation of the homologous transgene DNA rather than the transgene structure or the integration site. How this occurs is unknown but it is conceivable that DNA-RNA hybrids are formed (Wassenegger *et al.*, 1994). These structures might be good substrates of DNA-methyltransferases, like cruciform structures in DNA. Whether this

intriguing RdDM mechanism is just a response to certain nucleic acid interactions or a genuine regulatory mechanism to control gene expression by epigenetic changes remains to be resolved.

Although viroid RNA induces methylation of viroid transgenes which may spread into the promoter region and cause transcriptional silencing, it does not result in a detectable post-transcriptional silencing (and as a result viroid resistance) as is observed for the plus-RNA viruses (PVX, PVY). It is not known why, but one possibility is that the viroid RNAs cannot be reached for degradation as they reside in the nucleus (Harders *et al.*, 1989). At present it is not known if DNA methylation induced by viroid RNA is an exception or that other (abundant) RNAs can do the same. This interesting possibility was raised to explain some of the silencing phenomena involving methylated transgenes (Wassenegger *et al.*, 1994).

**Elimination of homologous transcripts.** If the PTGS mechanism is induced according the RNA threshold model it implies that cells are able to measure levels of specific RNAs. If this activity functions in normal cells monitoring RNA levels from endogenous genes, one would expect that it also establishes and maintains a certain steady-state RNA level in transgenic cells. This is not the case: the vast majority of homologous RNAs are degraded. Either such an RNA-control activity does not exist in normal cells or it is modified by the products of the transgenes, perhaps as a sort of defence mechanism against the possible harmful effects of these 'foreign' DNAs.

We can only speculate about the nature of this RNA degradation activity. Dougherty's group postulated a role for complementary RNAs (cRNA) synthesized by the plant-encoded RNA-dependent RNA polymerase (RdRP) in the cytoplasm (Lindbo *et al.*, 1993; Dougherty and Parks, 1995). RdRP from tomato was recently characterized (Schiebel *et al.*, 1993a) and it was shown that *in vitro*, this enzyme synthesizes small RNAs from RNA templates (Schiebel *et al.*, 1993b). It is hypothesized that *in vivo* these cRNAs hybridize to complementary mRNAs which are then degraded by double-stranded RNA specific RNases. The appeal of the RdRP-cRNA model is that it explains the strong sequence specificity of PTGS.

In the case of *chs* co-suppression in petunia, Metzlaiff *et al.*, (1996) detected considerable amounts of RNA degradation intermediates derived from the 3' half of the *chs* mRNA. Also smaller 3' end products were detected and the sequence of these smaller RNAs revealed that they were derived from transcripts of which a small segment was removed by cryptic splicing. This unusual splicing event and the finding that unspliced *chs* transcripts in *chs*-silenced tissues accumulate (Van Blokland *et al.*, 1996) suggest that the processing of primary transcripts is to some extent impaired. The response to the presence of these unusual transcripts is unknown but one may speculate that they can act as template for the RdRP.

**PTGS of endogenous genes and the presence of specific transgene loci.** Van Blokland *et al.* (1994) observed silencing of endogenous *chs* genes in petunia with promoterless *chs* transgenes and could not correlate silencing with a particular level of transgene expression. Apparently, the amount of

transgene product is not important, suggesting that the PTGS mechanism is activated in a different way. In this alternative way, the structure of the silencing T-DNA loci may play an important role. It is becoming clear that PTGS is often associated with multicopy T-DNA loci (Hobbs *et al.*, 1993; Dehio and Schell, 1994; Ingelbrecht *et al.*, 1994; Van Blokland *et al.*, 1994; English *et al.*, 1996; Stam and Kooter, unpubl. res.). It is, as yet, unknown how the organization of a transgene locus affects PTGS. There are at least two possibilities; the first is that a multicopy locus is prone to deliver the hypothetical aberrant RNA assumed to trigger the cytosolic RNA degradation machinery directly (see before). Obviously, this requires transcription of the transgenes. The aberrant nature of the RNAs might originate from read-through transcription, abnormal RNA processing, or transcription of a methylated template (Smith *et al.*, 1994; English *et al.*, 1996). When the RNA degradation machinery is activated, it eliminates all homologous RNAs. The second possibility applies only to cases where the genome contains an endogenous homologue (Fig. 1, far right). Given that barely-transcribed *chs* sense transgenes and promoterless transgenes are able to confer strong suppression of endogenous *chs* genes (Van Blokland *et al.*, 1994; Stam and Kooter, unpubl. res.) it is unlikely, though not excluded, that a transgene-derived RNA plays a key role in this PTGS process. In line with the RdRP-cRNA model (Dougherty and Parks, 1995), we think that aberrant RNAs might be derived from the endogenous *chs* genes. But how? One possibility is that it occurs as a result of ectopic DNA pairing (Jorgensen, 1992) between the transgene locus and the endogenous gene(s). Based on the detection of elevated levels of unspliced *chs* transcripts in nuclei containing silenced *chs* genes (Van Blokland *et al.*, 1996), this pairing event may have changed features of the endogenous gene leading to an impaired processing and/or transport of the RNAs (Fig. 1). These transcripts may be intrinsically unstable and rapidly degraded, or may act as aberrant RNA causing the degradation of other homologous RNAs. Not all transgene loci may be able to pair ectopically with an endogenous gene. An essential property seems that they are repetitive. Thus far, all the T-DNA loci that we have found to induce PTGS of *chs* contain two or more T-DNAs arranged as IRs (Stam and Kooter, unpubl. res.). In the case of IR-*chs*-silencing loci, sequences near the centre of the IR are more methylated than those at the borders, and when present, the CaMV-35S promoter is barely active. This methylation pattern might be formed due to the ability of an IR to form a cruciform which initiates at the centre. A property of a methylated repeat locus is that it can induce the methylation of an ectopic transgene copy, probably by DNA-DNA pairing, and inactivate it (see before). Given this possibility, it seems reasonable to assume that a methylated IR locus is able to interact with an endogenous gene thereby modifying its epigenetic state. Thus, regarding DNA-DNA interactions, TGS and PTGS seem to have features in common. Except, that they do not lead to transcriptional silencing of the endogenous genes, probably because the homology of the transgenes is usually confined to the coding region. There are no indications that the methylation status of the endogenous genes is changed.





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